Zinc Concentration in Esophageal Biopsy Specimens Measured by X-Ray Fluorescence and Esophageal Cancer Risk

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Background: **In rodents, zinc deficiency potentiates the effects of certain nitrosamines that act as esophageal carcinogens. Studies of the association between zinc and esophageal squamous cell carcinoma in humans have been hampered by plasma zinc homeostasis, which obscures individual differences in total zinc stores, and by the uncertainty regarding zinc bioavailability when estimating dietary zinc intake because phytate from whole grains effectively prohibits zinc absorption. By using baseline tissue biopsy specimens collected in a prospective observational study, we determined the association between incident esophageal squamous cell carcinoma and baseline element concentrations in tissue sections from residents of Linzhou, China, participating in a nutrition intervention trial.** ***Methods:*** **We used x-ray fluorescence spectroscopy to measure zinc, copper, iron, nickel, and sulfur concentrations in single 5-μm-thick sections from formalin-fixed, paraffin-embedded esophageal biopsy specimens collected in 1985 from 60 eventual case and 72 control subjects. Subjects were matched on baseline histology and followed for 16 years. We used Cox proportional hazards models to estimate the hazard ratios (HRs) and 95% confidence intervals (CIs) for the association between each element and risk of incident esophageal cancer. All statistical tests were two-sided.** ***Results:*** **The risk of developing esophageal cancer was much lower for subjects in the highest quartile of esophageal tissue zinc concentration compared with those in the lowest quartile (HR = 0.21, 95% CI = 0.065 to 0.68). The association was statistically significant across quartiles (P_{trend} = .015). Individuals in the highest quartile of sulfur concentration had a lower risk of esophageal cancer than individuals in the lowest quartile (HR = 0.29, 95% CI = 0.095 to 0.85), but the association across quartiles was not statistically significant (P_{trend} = .081). There was no association between copper, iron, or nickel concentrations and risk of esophageal cancer.** ***Conclusion:*** **High tissue zinc concentration was strongly associated with a reduced risk of developing esophageal squamous cell carcinoma. X-ray fluorescence spectroscopy can be used to assess relationships among concentrations of both nutritional and toxic elements and disease risk in banked tissue specimens. [J Natl Cancer Inst 2005;97:301–6]**

Zinc deficiency enhances the effects of certain nitrosamines (e.g., *N*-nitrosomethylbenzylamine) that act as esophageal carcinogens in rodents *(1,2)*. The relationship between zinc deficiency and nitrosamine carcinogenicity has been well studied in rodents with regard to cell proliferation *(3)*, P450-dependent metabolism of nitrosamines *(4)*, alkyl guanine DNA methyltransferase activity *(5)*, and the anticarcinogenic impact of zinc replenishment *(6)*. These studies demonstrate that the mechanisms by which zinc deficiency increases the incidence of esophageal carcinogenesis occur locally in the target tissue and are not the result of alterations in carcinogen metabolism at other sites, such as the liver. Thus, to study the relationship between zinc and the risk of developing esophageal squamous cell carcinoma in humans, the zinc concentration in esophageal tissue should be measured directly. One method that can be used to measure zinc concentrations in tissues directly is x-ray fluorescence spectroscopy *(7)*, in which a sample is bombarded with high-intensity x-rays that cause elements to fluoresce with a characteristic energy signature. X-ray fluorescence spectroscopy can nondestructively measure multiple-element concentrations in very small amounts of tissue, such as single 5-μm-thick sections from endoscopic biopsy specimens.

Because x-ray fluorescence spectroscopy directly measures actual tissues levels of elements, it has advantages over other methods of measuring zinc levels, such as quantifying serum zinc levels or estimating dietary zinc intake. Serum zinc concentrations are maintained homeostatically, and thus, serum zinc is a weak marker of zinc status in humans *(8)*. Estimating zinc intake on the basis of nutrient density in diet is complicated by the dramatic differences in zinc bioavailability created by other dietary constituents. For example, phytate in whole grain efficiently prohibits dietary zinc uptake *(9)*.

People who consume relatively little meat and large quantities of whole grain are more likely to be zinc deficient than those who eat more meat and more refined grains *(9)*. This dietary pattern (low meat, high whole grain) is seen in residents of Linzhou (formerly Linxian), People's Republic of China. Previous studies in Linxian found that zinc levels in patients with esophageal squamous cell cancers are lower than those in control subjects, in both serum (81 and 91 μg/dL, respectively) and esophageal tissue (81 and 97 μg/g [dry weight], respectively) *(10,11)*. Residents of

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Linzhou have some of the highest rates of esophageal squamous cell cancer and gastric cardia adenocarcinoma in the world, with more than 100 cases per 100 000 people per year *(12)*. Two trials, the Nutrition Intervention Trials, were carried out in Linzhou *(13)*, and a subset of the subjects enrolled in the Dysplasia Trial *(13)* underwent endoscopy with biopsy at the study baseline in 1985. These subjects have been followed through 2001.

In a prospective observational study, we used x-ray fluorescence to measure zinc, copper, iron, nickel, and sulfur levels in esophageal biopsy specimens from 60 subjects who developed esophageal squamous cell carcinoma during 16 years of follow-up and from 72 subjects, matched on worst baseline histology, who did not develop esophageal cancer.

Subjects and Methods

Subjects

In 1985, as part of the baseline evaluation for the Dysplasia Trial *(13)*, 3318 subjects completed a demographic questionnaire and a subset of 440 individuals had endoscopy and biopsy. All subjects provided oral informed consent, and the study was approved by the Institutional Review Boards of the U.S. National Cancer Institute and the Cancer Institute, Chinese Academy of Medical Sciences. Esophageal biopsy specimens contained only esophageal epithelium without lamina propria. All trial subjects were followed to ascertain vital status and incidence of cancer through May 31, 2001. Case ascertainment *(13)* and cancer definition *(14)* were as described previously, and all cases were verified by an international panel of experts. Among the participants who received endoscopy, 88 subjects developed esophageal squamous cell carcinoma, 60 of whom had sufficient tissue remaining in their biopsy specimens for x-ray fluorescence analysis. From the other 352 subjects who had endoscopy, we selected a group of 88 subjects who had not developed esophageal squamous cell carcinoma as of May 31, 2001 and matched them to the case subjects on worst baseline histology. Of the 88 control subjects, 72 had sufficient tissue remaining in their biopsy material for analysis. X-ray fluorescence measurements were therefore completed on a total of 132 samples.

X-Ray Fluorescence Measurements of the Tissue Concentration of the Elements

A single 5-μm-thick section was cut from each tissue block and mounted on 3525 Ultralene XRF film (SPEX CertiPrep, Metuchen, NJ). X-ray fluorescence measurements were carried out at beamline 2-BM of the Advanced Photon Source (Argonne National Laboratory, Argonne, IL). X-rays of 10.5 keV energy were focused to a 100-micron-diameter spot on the specimen. X-ray fluorescence emission was collected by an energy-dispersive silicon drift detector. Each experimental tissue was measured at two randomly selected sites within the tissue and at a single spot within the embedding wax outside the tissue. X-ray fluorescence emission spectra were collected for 200 seconds. In addition to measuring the experimental tissues, we measured a single section on five separate occasions in the same spots to assess the coefficient of variation for these measurements. Finally, we measured NIST standards NBS 1832 and NBS 1833 (National Institute of Standards, Gaithersburg, MD) to allow quantitative estimates of element concentrations.

The intensity of the x-ray fluorescence for each of the different elements was determined by fitting the elemental peaks in the x-ray fluorescence spectra to modified Gaussian functions *(7)*. The measured sample intensities were converted to element concentrations (nanograms/cm^2) by comparison with a calibration curve. The calibration curve was calculated from measurement of the NIST 1832 and 1833 standard reference materials by taking into account, for each chemical element of interest, the corresponding photoelectric absorption cross sections *(15)* based on fluorescence yield, absorption by the Beryllium window of the energy-dispersive fluorescence detector, and absorption by a dead layer on the detector. Individual experimental tissue concentrations for each element were derived by averaging the two tissue measurements and then subtracting the measurement in the embedding wax. After graphing the data, we found that the distribution of the tissue concentrations had a long right tail (i.e., the data were not normally distributed); we then log transformed the data, after which the distribution of the concentrations was approximately normal. Therefore, all analyses used log-transformed data. Of the 660 experimental sample measurements, only five measurements (one zinc, two nickel, one copper, and one iron) were more than 4 standard deviations from the geometric mean and had no other values nearby. We classified these measurements as outliers and excluded them from further analyses.

Assay Reliability

Using the five repeated measurements of the same section, we estimated the coefficient of variations to be 0.5% for zinc, 6% for copper, 3% for iron, 29% for nickel, and 3% for sulfur. The coefficient of variation for the four elements other than nickel were excellent, and that for nickel was acceptable. The lower coefficient of variation for nickel may reflect its low absolute concentration in our tissue sections. We examined the distribution of the differences between the two measurements of each experimental sample and found these to be normally distributed, and the mean of the differences did not statistically significantly differ from zero. Because the median ratios of the tissue-to-wax concentrations (i.e., foreground to background) were 28 for zinc, four for copper, three for iron, six for nickel, and two for sulfur, we had sufficient element concentrations above the background level in the wax to be unconcerned about potential contamination.

Statistical Analysis

Demographic data for case and control subjects was compared using chi-square tests for categorical variables and the Wilcoxon rank-sum test for age. The Wilcoxon rank-sum test was also used to compare element concentrations between cases and control subjects. Pearson's correlation coefficient was used to examine the correlation between element concentrations.

In our other analyses, we examined associations between cancer risk and three different constructs for the tissue concentrations. First, we centered and standardized the continuous variable (i.e., the element concentration) by subtracting the median value and dividing by the average size of the two central quartiles of the control values (0.5 × interquartile range). Second, we created quartile variables on the basis of the actual distribution of the tissue element concentrations from the control subjects. Third, we created an ordinal quartile trend variable in which the value

from each subject was 1, 2, 3, or 4 depending on the quartile in which the subjects' value was assigned. For each construct, we used linear regression to examine the association between the log-transformed element concentrations and potential confounding factors such as age, sex, smoking status, and consumption of alcohol. We used the Wilcoxon rank-sum test to test the univariate association between tissue element concentration and esophageal cancer. To estimate within-quartile nonparametric survival curves and hazard ratios (HRs) in the Cox proportional hazards model, we used the estimators of Mark *(16,17)* as implemented in the R-software *(18)* by Katki and Mark *(19)*. These estimators are weighted versions of the usual Nelson-Aalen and maximum partial-likelihood estimators *(20)* of the cumulative hazard ratios and hazard ratios, respectively. The weights account for the outcome by baseline histology-specific sampling fractions and are required to produce unbiased estimators *(16,17)*. The nonparametric estimates of survival curves were obtained from the cumulative hazards by exponentiating the negative of the cumulative hazard estimates *(16,17,20)*. These estimates are asymptotically equivalent to Kaplan-Meier estimates and are hereafter referred to as Kaplan-Meier survival curves adjusted for sampling fractions. All estimates of hazard ratios come from Cox proportional hazards models adjusted for age (continuous variable) and with indicator variables for sex; ever smoking (regular use for ≥6 months); drinking (any alcohol in the preceding 12 months); and mild, moderate, and severe dysplasia at baseline. Using no variable or a single variable (i.e., any dysplasia) to correct for histology produced results essentially similar to those reported. To test for deviations from the proportional hazards assumption, we fit Cox proportional hazards models by using spline parameterizations of the hazard ratios that allowed them to vary flexibly with time. No time trends were detectable. All *P*-values were two-sided and derived from the Wald test. Tests for trend were the usual one-degree of freedom test for log-linearity and used the ordinal classifications of element concentration quartile.

Table 1. Subject characteristics by case status*

Characteristic	Control subjects (n = 72)	Case subjects† (n = 60)
Median age, y, (interquartile range)	55 (49–59)	55 (50–59)
Sex, % female	53%	58%
Smoking, ever regularly for ≥6 mo, % yes	28%	28%
Alcohol drinking, any in the previous 12 mo, % yes	22%	12%
Family history of cancer, % yes	51%	50%
Histology, n (%)		
Normal	19 (27%)	16 (27%)
Esophagitis	24 (33%)	20 (33%)
Dysplasia‡	29 (40%)	24 (40%)

*Endoscopy and biopsy collection was completed in 1985. All subjects were cancer free at study baseline (1985). Incident esophageal squamous cell carcinomas occurred between 1985 and 2001. Subjects were matched on worst baseline histology.

†Control and case subjects were compared by using the chi-square test for categorical variables and the Wilcoxon rank-sum test for age. All *P* values were >.05.

‡Subjects with dysplasia were further matched on whether they had mild, moderate, or severe dysplasia.

Table 2. Baseline medians (interquartile ranges) of esophageal tissue element concentrations according to future case status among residents of Linzhou, China

Element	Element concentration, ng/cm^2		*P**
	Control subjects (n = 72)	Case subjects (n = 60)	
Zinc†	57 (47–108)	44 (30–75)	.008
Copper	9.5 (0.22–20.3)	8.3 (1.6–13.7)	.22
Iron	9.3 (5.4–16.5)	10.0 (5.6–19.5)	.78
Nickel	0.88 (0.54–1.39)	0.92 (0.53–1.60)	.82
Sulfur	815 (619–1000)	752 (561–916)	.13

**P* values come from the Wilcoxon rank-sum test and the normal approximation *P*.

†Tissue element concentrations were measured by using x-ray fluorescence.

Results

Subject Characteristics

Subject characteristics are presented in Table 1. Subjects were similar in all categories, with the exception of alcohol consumption, which was less frequent among case subjects. Less than 25% of the Linzhou population consumed any alcohol, and among users, the amount consumed was very low. This distribution of alcohol consumption is similar to the cohort from which the subjects were drawn.

Tissue Element Concentrations

The medians and interquartile ranges for baseline levels of each of the measured elements (for case and control subjects stratified by future cancer incidence) are given in Table 2. Subjects who later developed esophageal cancer had statistically significantly lower baseline esophageal tissue concentrations of zinc than those who did not develop this cancer (44 versus 57 ng/cm^2, *P = .008)*. Levels of copper (8.3 versus 9.5 ng/cm^2, *P = .22)*, iron (10.0 versus 9.3 ng/cm^2, *P = .78)*, nickel (0.92 versus 0.88 ng/cm^2, *P* = .82), and sulfur (752 versus 815 ng/cm^2, *P = .13)* did not differ statistically significantly between case and control subjects, respectively.

We used linear regression to assess whether age, sex, ever smoking for ≥6 months, consumption of alcohol within the last 12 months, or family history of cancer was associated with the concentration of each of the elements in the tissue sections. The association between any consumption of alcohol and zinc concentration was the only statistically significant relationship (*P* = .036). Including terms for either baseline histology or cancer outcome in the linear regression models did not change these results.

Tissue Element Concentrations and Cancer Risk

We examined the relationship between incident esophageal squamous cell carcinoma for study participants stratified by quartile of tissue zinc concentration (Fig. 1). Approximately 90% of individuals in the highest zinc quartile were alive without esophageal cancer after 16 years (i.e., at the end of follow-up). By contrast, only 65% of individuals in the lowest zinc quartile were alive without esophageal cancer after 16 years. The individuals in the second and third quartile of zinc concentration show intermediary disease-free survival rates of 85% and 80%, respectively.

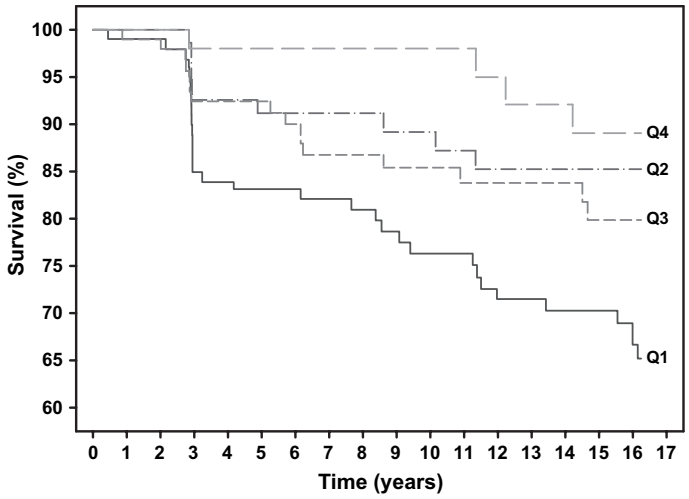

Fig. 1. Percentage of incident esophageal squamous cell carcinoma-free survival among residents of Linzhou, People's Republic of China, according to quartile of baseline tissue zinc level. The curves are weighted versions of Kaplan–Meier curves *(16,17)*, with the weights accounting for the sampling scheme specified by the study design (*see* "Subjects and Methods"). Q1 through Q4 refer to tissue zinc quartiles 1 through 4, with Q1 having the lowest concentrations of zinc and Q4 having the highest concentrations. Individuals in Q1 had a much lower probability of being disease-free at the end of follow-up than individuals in Q4. During 16 years of follow-up, the numbers of incident esophageal squamous cell carcinomas among individuals in each quartile of zinc (lowest to highest) were 31, 9, 15, and 5.

The covariate-adjusted hazard ratios and 95% confidence intervals (CIs) for the associations between tissue element concentrations and esophageal squamous cell carcinoma are given in Table 3. When we used the tissue concentration of zinc as a continuous variable, we found that, for each concentration increase equivalent to the average size of the two central quartiles (i.e., approximately 25% of the distribution), the risk of esophageal squamous cell carcinoma decreased by 26% (HR = 0.74, 95% CI = 0.56 to 0.97). We also examined the risk of esophageal cancer associated with tissue zinc concentrations by quartile and found that individuals in the highest quartile of tissue zinc concentration were at a statistically significantly lower risk of developing esophageal cancer than individuals in the lowest quartile (HR = 0.21, 95% CI = 0.065 to 0.68). Compared with individuals in the lowest quartile, individuals in quartiles two and three also had lower risks of developing esophageal cancer (quartile 2, HR = 0.48, 95% CI = 0.17 to 1.31; quartile 3, HR = 0.62, 95% CI = 0.28 to 1.38), with the ordering of the point estimates identical to that found in the survival curves (Fig. 1). Finally, there was a statistically significant overall trend between decreasing risk of esophageal squamous cell carcinoma and increasing tissue zinc quartile (P_{trend} = .015).

We also examined the relationships between other measured tissue element concentrations and the risk of esophageal squamous cell carcinoma. For copper, using the trend test, individuals with higher baseline concentrations had a decreased risk of esophageal cancer, but this association was not statistically significant (P_{trend} = .22). For sulfur, although neither the continuous estimate (P = .097) nor the trend test (P_{trend} = .081) was statistically significant, individuals in the highest quartile had a statistically significantly lower risk of esophageal cancer than individuals in the lowest quartile (HR = 0.29, 95% CI = 0.096 to 0.85). Tissue zinc and sulfur concentrations were moderately correlated (r = 0.5). After simultaneously adjusting the models for zinc and sulfur concentrations for each other, the inverse association with risk of esophageal cancer and element concentration for sulfur decreased (approximately 20% closer to the null value of 1) and that for zinc increased (approximately 5% further from the null value), but these changes were not substantial.

Discussion

This study is, to our knowledge, the first prospective study to examine the association between tissue elemental zinc levels and esophageal squamous cell carcinoma. Our finding, that zinc concentration is inversely associated with risk of incident esophageal squamous cell carcinoma, strengthens the hypothesis that zinc deficiency is a contributing factor to the development of esophageal squamous cell carcinoma in humans. We developed a method to accurately measure element concentrations in the small esophageal biopsy specimens we had available and evaluated the relationship between the concentrations and the risk of developing esophageal squamous cell carcinoma over the following 16 years. We compared subjects in the highest quartile

Table 3. Hazard ratios and 95% confidence intervals* between esophageal tissue element concentrations and incidence of esophageal squamous cell carcinoma among residents of Linzhou, China

	Continuous variable†		Quartile‡				
			1	2	3	4	
Element	HR (95% CI)	P§	Referent	HR (95% CI)	HR (95% CI)	HR (95% CI)	P_{trend}§
Zinc	0.74 (0.56 to 0.97)	.028	1.0	0.48 (0.17 to 1.31)	0.62 (0.28 to 1.38)	0.21 (0.065 to 0.68)	.015
Copper	0.88 (0.73 to 1.05)	.16	1.0	0.90 (0.39 to 2.09)	0.71 (0.29 to 1.70)	0.55 (0.19 to 1.56)	.22
Iron	1.06 (0.88 to 1.29)	.53	1.0	1.05 (0.38 to 2.86)	0.91 (0.37 to 2.29)	1.48 (0.57 to 3.89)	.50
Nickel	1.03 (0.85 to 1.24)	.80	1.0	0.47 (0.17 to 1.29)	0.74 (0.29 to 1.85)	0.96 (0.35 to 2.61)	.83
Sulfur	0.84 (0.69 to1.03)	.097	1.0	0.51 (0.19 to 1.32)	0.81 (0.32 to 2.01)	0.29 (0.096 to 0.85)	.081

*All estimates of hazard ratios (HRs) and 95% confidence intervals (CIs) come from Cox proportional hazards models weighted for the sampling fraction by baseline histology in the underlying cohort. All models were adjusted for continuous age, sex, smoking history, and history of alcohol consumption and with indicator variables for mild, moderate, and severe dysplasia.

†For continuous estimates, the concentrations were centered on the median and standardized to the average sizes of the two central quartiles. The values in ng/cm^2 are as follows: Zn = 30.8, Cu = 9.1, Fe = 5.5, Ni = 0.43, and S = 190. Outliers were removed from some of the continuous estimates (*see* "Subjects and Methods").

‡Quartile cut points were based on the distributions of tissue concentrations among the control subjects and can be read from the median (interquartile range) given in Table 2 for control subjects.

§The P values for the continuous variable and the ordinal trend tests come from the Wald test.

of esophageal zinc concentration with those in the lowest quartile and found that the subjects with higher zinc concentrations were at greatly reduced risk of developing esophageal squamous cell carcinoma (HR = 0.21).

Our results are different from those obtained from an intervention trial conducted in the same subjects, in which they received zinc as part of a multivitamin supplement and in the companion General Population Trial, in which one group of subjects received zinc and retinol supplements *(14,21)*. None of these trials showed protective effects of zinc supplements against esophageal squamous cell carcinoma incidence. In this observational study, by contrast, in which we examined zinc status by using esophageal tissue concentration, we found that higher zinc concentration in esophageal tissue was associated with a lower risk of esophageal squamous cell carcinoma. This same pattern—i.e., an association between baseline nutritional status and cancer rate in an observational study but no association in a randomized trial with the same subjects—was also seen for selenium and α-tocopherol in this Chinese population *(21,22)*. There are several potential explanations for the discrepancy in findings between the observational study of baseline nutrient status and the randomized trial of nutrient supplementation in the same subjects. For zinc, these possibilities include that the trial supplementation period was too short and/or the dose was too low, that the effects of lifelong zinc deficiency on carcinogenesis are not remediable by zinc supplementation later in life, that the form and method of zinc supplementation was ineffective, or that the presence of other elements such as iron *(23)* in the multivitamin supplement inhibited zinc absorption.

Our study is unique in that it examined elements rarely included in other studies, regardless of design. Copper has been examined in ecologic studies of dietary intake *(24)*, but to our knowledge, prospective studies have not been reported. High nail concentrations of iron have been associated with increased risk of esophageal cancer in a U.S. case-control study *(25)*. Metallic nickel and certain nickel compounds are reasonably anticipated to be human carcinogens *(26)* but have not been associated with esophageal cancer in human studies. The role of sulfur in esophageal cancer risk is difficult to assess because of its ubiquitous role in biology. Our results for copper, iron, nickel, and sulfur are somewhat equivocal, and the importance of these elements in the risk of developing esophageal squamous cell carcinoma will require further study.

Our study has several strengths. We used prospectively collected biologic samples from subjects nested in a cohort with essentially complete follow-up and disease status ascertainment. We measured zinc status by using target tissue concentrations, which is arguably the best method for assessing associations being zinc and the risk of disease.

Our study also has limitations. First, x-ray fluorescence cannot provide information on the form of the element (e.g., valence state) and this may be important for some toxic metals, such as arsenic. Second, x-ray fluorescence provides only total element concentrations without regard to whether the element is bound or complexed, possibly limiting the utility of the technique for certain element hypotheses (e.g., iron). Finally, the study was relatively small. Because it is the first report of a prospective association between zinc tissue concentration and risk of esophageal cancer, it will require confirmation in an independent study.

Many prospective cohort studies and medical institutions have tissue banks that can provide samples for studies of cancer or other diseases that are similar in design to our study. Prospectively collected tissue samples are precious resources that can be used sparingly to address important hypotheses. But these hypotheses can be investigated using only analytical techniques with sufficient sensitivity and reproducibility for the small biological samples available. One advantage of our analytical method is the high sensitivity and multiple-element capabilities of x-ray fluorescence, which required only a single tissue section. Our study, which used x-ray fluorescence, provides a model for other studies of potential associations between nutritional or toxic elements and the risk of subsequent disease.

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Notes

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